Research Paper

Isolation of nine *Phytophthora capsici* pectin methylesterase genes which are differentially expressed in various plant species

Peiqian Li[1], Baozhen Feng[1], Hemei Wang[1], Paul W. Tooley[2] and Xiuguo Zhang[1]

[1] Department of Plant Pathology, Shandong Agricultural University, Tai'an, P.R. China
[2] USDA, ARS, Foreign Disease-Weed Science Research Unit, 1301 Ditto Ave., Ft. Detrick, MD, USA

Phytophthora capsici causes damage on many plants species, and secretes various pectin methylesterases during all stages of infection. We identified nine *Pme* genes (*Pcpme* 1–9) from a genomic library of highly virulent *P. capsici* strain SD33 and further analyzed the expression pattern of nine genes on three hosts: pepper, tomato, and cucumber using qRT-PCR during all stages of infection. All nine genes were found to be differentially expressed in three host species in the course of *P. capsici* interaction. The expression levels of the respective genes increased from 1 to 7 dpi in pepper, while most genes presented a decreasing trend of expression from 1 to 5 dpi in tomato fruits. However, in both fruits peaks were reached at 7 dpi. In cucumber fruits, each gene showed minor expression levels from 1 to 3 dpi, exhibited definite peaks at 5 dpi, and then decreased from 5 to 7 dpi. Thus, evidence from our studies of *Pcpme* gene expression in different plants at a rang of time points suggests that the late stages of infection may represent the most critical time for *P. capsici* to successfully express or/and secret PMEs.

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Introduction

Phytophthora capsici Leonian was first described in 1922 on *Capsicum annuum* L. in New Mexico [1]. Originally, this pathogen was considered to be host-specific [2], but since then it has been identified worldwide and reported as a devastating pathogen on a range of solanaceous and cucurbitaceous hosts including pepper, cucumber, eggplant, squash, pumpkin, tomato, melon, and zucchini [3, 4]. *P. capsici* often causes root and crown rot, as well as stem, leaf, and fruit lesions. Sporangia and/or oospores develop in the lesions, resulting in fruit surfaces having a powdered-sugar appearance. Infected fruits quickly degrade, both in the field and postharvest [4, 5].

Cell wall degrading enzymes (CWDEs) play an important role in all the infection process of plant pathogens. The role of CWDEs was first reported by DeBary [6] and subsequently, a relationship has been observed between pathogenicity and the CWDEs production ability of plant pathogens [7]. The CWDEs are often observed during the initial stages of pathogenesis and have been suggested to be instrumental in host penetration. Since CWDEs are in addition, always found later in the infection process, they may also be involved in further steps of infection. In fact, plant cell walls that are degraded by CWDE activity, may facilitate pathogens growth by providing nutrients. Pectin degrading enzymes are among the numerous CWDEs produced by plant pathogens. To degrade pectin, plant pathogens produce different types of pectinases during the infection process

Correspondence: Xiuguo Zhang, Department of Plant Pathology, Shandong Agricultural University,Tai'an, 271018, China
E-mail: zhxg@sdau.edu.cn
Phone: 086-0538-8246350
Fax: 086-0538-8249095

that are classified by their substrates and mode of action on the pectin polymer [8]. Unesterified pectate polymers can be degraded by polygalacturonase (PG; EC 3.2.1.15) using hydrolytic cleavage, and by pectate lyase (PL; EC 4.2.2.2) using β-elimination cleavage and the formation of a double bond in one of the resulting galacturonate residues. Pectin methylesterase (PME; EC 3.1.1.11) removes the methyl group from esterified galacturonic acid residues in pectin chains [9]. Our previous studies revealed that the CWDEs were contributing to virulence of *P. capsici* during the course of infection [10, 11] in a manner similar to that of CWDEs from bacterial [12, 13] and fungal pathogens [14–17]. These studies have shown that pectinases, which are among the CWDEs secreted by *P. capsici*, play an important role in the infection process. Many hosts of *P. capsici* including many dicotyledons, contain high levels of pectin in their cell walls [3, 4].

Pectin is one of the most important plant barriers to be overcome in establishing infection of plants by *P. capsici*, which shows many similarities with filamentous fungi in the infection process [18]. Penetration by *P. capsici* mycelium often takes place at the host cell wall, followed by growth within the host and production of sporangia on the surface of the diseased tissue which occurs with high frequency under warm and wet conditions [19]. Sporangia and zoospores represent secondary asexual forms of inoculum produced on infected plants, and can be responsible for rapid disease progression.

Little is known of the activity of pectin methylesterase in *P. capsici* except that it was first detected during the infection process and its activity was found during all steps of infection [10]. In these studies, we wish to characterize expression patterns that may be observed in different hosts, which could be beneficial for further analysis of *P. capsici* infection and shed light on why *P. capici* has a broad host range. Such information will enhance our understanding of the molecular mechanisms related to *P. capsici* infection, and contribute to our understanding of the various functions occurring during interaction with different host plants. We isolated and identified nine novel *Pcpme* genes (*Pcpme*1–9) from a genomic library of *P. capsici* [11], and performed a detailed study on expression of these nine *pme* genes in plant tissue inoculated with *P. capsici*. Our findings indicate that products of these nine *Pcpme* genes facilitate the decomposition of host pectin. The results also allow a number of predictions to be made regarding *Pcpme* gene expression in the pepper following inoculation with *P. capsici*.

Materials and methods

P. capsici strain, isolation of *Pcpme* genes and sequence analysis

Strains of *P. capsici* were isolated from blighted pepper plants collected from the field in China and identified as *P. capsici,* as described by Waterhouse [20]. High-virulent *P. capsici* strain SD33 [5, 10] was used in these studies, and genomic DNA was extracted as previously described [21]. A genomic library of *P. capsici* was constructed as previously described [11] and screened using five pairs of degenerate primers (Table 1) designed ba-

Table 1. The primers used for gene cloning in this study.

Primer	Sequence	Purpose
Nsp	5′-CCA(T/G)GGACGGCC(C/A)AG(A/C)TAGGCGG-3′	*Pcpme*1
NAsp	5′-TCGA(C/T)TTT(G/A)T(A/C)TTCGGTACCAAGGCCG-3′	*Pcpme*1
P230	5′-CCGGG(A/T/G/C)GT(G/C)TACCA(A/C)GAGC-3′	*Pcpme*2
P920	5′-CC(A/G/C)(G/C)(G/A/T)GTTGTTGAACTCCTTG-3′	*Pcpme*2
P650	5′-AGGCGTGGTT(C/T)GA(G/A)TCGTGCG-3′	*Pcpme*3
P920	5′-CC(A/G/C)(G/C)(G/A/T)GTTGTTGAACTCCTTG-3′	*Pcpme*3
P650	5′-AGGCGTGGTT(C/T)GA(G/A)TCGTGCG-3′	*Pcpme*4
P920	5′-CC(A/G/C)(G/C)(G/A/T)GTTGTTGAACTCCTTG-3′	*Pcpme*4
Sp1	5′-TTCCAGGGACGGCCAAGCTAGGC-3′	*Pcpme*5
Asp	5′-TCGATTTTATATTCGGTACCAAGGCCG-3′	*Pcpme*5
P230	5′-CCGGG(A/T/G/C)GT(G/C)TACCA(A/C)GAGC-3′	*Pcpme*6
AP650	5′-CGCACGA(C/T)TC(G/A)AACCACGCC-3′	*Pcpme*6
P650	5′-AGGCGTGGTT(C/T)GA(G/A)TCGTGCG-3′	*Pcpme*7
P920	5′-CC(A/G/C)(G/C)(G/A/T)GTTGTTGAACTCCTTG-3′	*Pcpme*7
P650	5′-AGGCGTGGTT(C/T)GA(G/A)TCGTGCG-3′	*Pcpme*8
P920	5′-CC(A/G/C)(G/C)(G/A/T)GTTGTTGAACTCCTTG-3′	*Pcpme*8
P445	5′-TGTACAACCTCAA(C/T)(A/G)T(C/G)GCC-3′	*Pcpme*9
P920	5′-CC(A/G/C)(G/C)(G/A/T)GTTGTTGAACTCCTTG-3′	*Pcpme*9
9RAFP	5′-GCCTGGTTTGAGTCGTGCGACTTGGAGT-3′	*Pcpme*9 3′RACE
9RANP	5′-TCGGCAAAGGTGCCGTCACTGCTAATGG-3′	*Pcpme*9 3′RACE

sed on conserved sequences of other *pme* genes [22–24]. *Pcpme* genes were screened from the genomic library according to the procedure described by Liu *et al*. [25]. Clones containing the *pme* gene were sequenced. For the incomplete sequences, 5′ RACE and 3′ RACE were performed on 1 μg of total RNA of the strain using the SMART™ RACE cDNA Amplification Kit (Clonetech) according to the manufacturer's protocol. Primers used for RACE are also listed in Table 1. The PCR products were verified by sequencing. To verify the *pme* gene amino acid sequence, sequence data was analyzed using appropriate programs in the GCG software package (Genetics Computer Group, Wisconsin Package Version 10.0). Nucleotide and amino acid sequence homology searches were compared with the sequences in the NCBI-BLAST program (http://www.ncbi.nlm.nih.gov/). Most of the available complete PMEs amino acid sequences including those of straminopilous pathogens and fungi were multialigned using Clustal X 1.83 [26] and GeneDoc (version 2.6.002) [27].

Fruit inoculation

For inoculation experiments, the strains were grown on oatmeal agar (100 g oatmeal, 20 g agar, 1000 ml of water), and induced to produce sporangia and zoospores as described [5, 28]. Inoculum density was adjusted with sterile water to give a suspension containing 1×10^5 zoospores per milliliter. Immature green pepper fruits (*Capsicum annuum* L. var. *grossum* L.), tomato fruits (*Lycopersicon esculentum* Mill.), and cucumber fruits (*Cucumis sativus* Linn.) were grown in the greenhouse, harvested early in the morning, and transferred immediately to the laboratory. All fruits were free of physical injuries and the inoculation process used was identical for peppers, tomatoes, and cucumber fruits. Prior to inoculating, the fruit surfaces were disinfested with 70% ethanol, and tissue was removed (1 cm deep) with a cork borer (0.7 cm diameter). A small quantity of sterile cotton that was dipped in 1×10^5 zoospores per milliliter for 30 min was placed on the wounded sites of fruits, which were then placed in a culture box at 28 °C, 95% RH with a 12 h photoperiod. The sector about 1–2 cm surrounding the wounded sites was collected at 1, 3, 5 and 7 d after inoculation and stored at –20 °C for RNA extraction.

RNA extraction and primer design for real-time RT-PCR

RNA from infection fruits was extracted using an Rneasy plant Mini Kit (Qiagen, Maryland, USA) according to manufacturer's instructions, followed by RNase-free DNase treatment (Takara, Japan). RNA concentrations were quantified by a spectrophotometer (spectraMax plus 384; Molecular Devices, Sunnyvale, CA, USA) and reverse transcription was performed using a RETRO Script Kit (Ambion) according to manufacturer's instructions. Specific primer of each *Pcpme* gene was designed by avoiding conserved regions using Clustal X 1.83 [29]. 18S rRNA from three plant species was chosen as the internal control [30]. The specific primers for real time RT-PCR were designed using Primer Express 3.0 software (Applied Biosystems, Foster City, USA) (Table 2).

Real time PCR

Real time quantitative PCR was performed in the ICycler IQ real-time PCR detection system (Bio-Rad, Denmark) using SYBR primer Script RT-PCR kit (TakaRa, Japan). For PCR reactions, 2.5 μl of cDNA template was added to 12.5 μl of the 2 × SYBR Green PCR master mix, 800 nM of each primer and ddH_2O to a final volume of 25 μl. After a denaturation step at 95 °C 10 min, the cycle profile used was 10 s at 95 °C, 55 s at 60 °C, and 45 s at 72 °C for 45 cycles. All reactions were performed in triplicate, and negative controls (with no template) were included for each gene. The threshold cycle (C_T) values were determined automatically by the instrument, and the fold changes of each gene were calculated using the equation $2^{-\Delta\Delta C_T}$, where ΔΔCT = (CT target−CT 18S rRNA) Sample x – (CT target−CT 18S rRNA) Sample 1 [31]. In this study, sample 1 of each gene acted as the mock infection, whereas sample X was PCR production of respective *Pcpme* genes at two-day intervals from 1 to 7 dpi or one of the nine *Pcpme* genes.

Results

Nine Pcpme genes sequence and structure

Thirty-two screened clones from the genomic library were sequenced, and a database search confirmed that nine genes were identified. These nine complete sequences were homologous to fungi, plant and other straminopilous *pme* genes. These nine *Pcpme* genes were designated *Pcpme*1 to *Pcpme*9 (accession numbers in GenBank: EF596784, FJ213426-33). When multiple amino acid sequence alignment of these nine *Pcpme* genes and fifteen *Pcpme* genes (jgi/Phycaf7/5752, 5827, 14496, 18199, 25092, 29809, 66599, 66841, 70513, 76209, 76210, 76586, 81618, 114940, 117376) of *P. capsici* download from Joint Genomics Institute were performed, similarities ranged from 90.54% to 99.42%, and none of these fifteen *Pcpme* genes from JGI were identical to any of the nine novel *Pcpme* genes (presented in supplement-

Table 2. Primers used for real-time PCR (RT-PCR) assay.

Target	Primer	Sequence(5′-3′)	Amplicon (bp)
Pcpme 1	Forward	CAGGTGCTCATTTGGAAGTTGAA	210
	Reverse	TGTTGGCGATGTTGAGGTTGTA	
Pcpme 2	Forward	TTTACAACGAGCAGGTTTTGGTT	205
	Reverse	GTACACCTTGACGTTGTCCGA	
Pcpme 3	Forward	AACGTACCAAAAGCAAGTCACA	174
	Reverse	CAGAGTGGTGGTGTAGTCGTT	
Pcpme 4	Forward	TTTCCAGGGTTGTATCTAGAGCA	170
	Reverse	CAAGTCGTTGCGGTTGTTCTT	
Pcpme 5	Forward	CAGGTGCTCATTTCGAAGTTGAA	212
	Reverse	TGTGTTGGCGATGTTGAGGTT	
Pcpme 6	Forward	CCGAAGTTAGCTGGACCGTT	172
	Reverse	ACTCCATTGCGACACTTGAGA	
Pcpme 7	Forward	ACGACATCCTACGCTTCCAA	153
	Reverse	TGTTGGCGATGTTGAGGTTGTA	
Pcpme 8	Forward	CGTACGCTGCCAACCAAGT	155
	Reverse	CGGTAGGATTGGCGACGTTA	
Pcpme 9	Forward	CGAGCACACGGTCTTCATGT	155
	Reverse	AAATCCCTTTGAGCCATTGTGT	
Capsicum annuum	Forward	TTTCGGTCCTATTACGTTGG	121
18S rRNA	Reverse	TTCGCAGTTGTTCGTCTTTC	
Lycopersicon esculentum	Forward	AAATGCCAGTCCACGTCGA	158
18S rRNA	Reverse	GGTAATCCCGCCTGACCTG	
Cucumis sativus	Forward	GTGCAACAAACCCCGACT	131
18S rRNA	Reverse	AATGATCCGTCGCCAGCA	

ary data Fig. S1). These nine *Pcpme* genes and other fifteen *Pcpme* genes from JGI may have been derived from different *P. capsici* strains or different mating types. On the basis of alignment of the nine *Pcpme* amino acid sequences and other PMEs from oomycete fungi, five conserved sequence segments (73_GxYxE, 157_QAVAT, 179_QDTV, 201_DFVFG, and 257_LGRPW) and six strictly conserved residues (Gly73, Asp180, Gly198, Asp201, Gly205, Arg259 and Trp261) existed in these nine novel *Pcpme* genes (Fig. 1). These conserved segments have been found in most other reported PMEs belonging to the carbohydrate esterase family CE-8 [20]. Each *Pcpme* gene also has two aspartic acid residues (Asp180 and Asp201) that are regarded as highly conserved in the active-site region in most of the PMEs [32, 33]. Moreover, three additional highly conserved segments (168_YGFYAC, 210_AWFESCD, and 239_YVFNNARVF) were only found in amino acid sequences of *Pcpme* genes and other well-known straminopilous pathogen PMEs.

The ORF of these nine *Pcpme* genes varies from 1029 to 1047 bp, and encodes the polypeptide of numbered amino acid residues varying from 338 to 349. They all contain a signal peptide of amino acid residues ranging in number from 16 to 20. Otherwise, there are a number of potential *N*-linked glycosylation sites on amino acid sequences as shown in Table 3. None of the *Pcpme* genes had an intron.

Expression of the nine Pcpme genes during infection of pepper fruits

Pepper fruits exhibited increasingly severe lesions or decay from 1 to 7 dpi (data not shown). The expression levels of nine *Pcpme* genes were estimated by qRT-PCR. Three additional transcripts were selected and evaluated with regards to the stability of their gene expression among different RNA samples, in an attempt to select at least one appropriate internal control. After analysis as previously described [34], 18s rRNA was selected as an appropriate internal standard based on low variation among the different samples (data not shown).

The expression patterns of the nine selected *Pcpme* genes were investigated at two-day intervals from 1 to 7 dpi (Fig. 2). Although different expression levels among the *Pcpme* genes were elicited in inoculated tissues up to 7 dpi, the expression levels of each gene showed an increasing trend in the infection process and eventually reached definite peaks at 7 dpi. *Pcpme*1, *Pcpme*2, *Pcpme*5, *Pcpme*7, and *Pcpme*8 exhibited lower expression from 1 to 3 dpi (Fig. 2A, B, E, G, and H) then showed an obvious increase at 5 dpi, peaking at 7dpi. In contrast, *Pcpme*3 and *Pcpme*9 were detected at lower levels up to 5 dpi (Fig. 2C and I). And the expression levels of *Pcpme*4 and *Pcpme*6 appeared to be minimal at 1 dpi and showed an increase from 3 to 5 dpi (Fig. 2D and F). In summary, the nine *Pcpme* genes showed in-

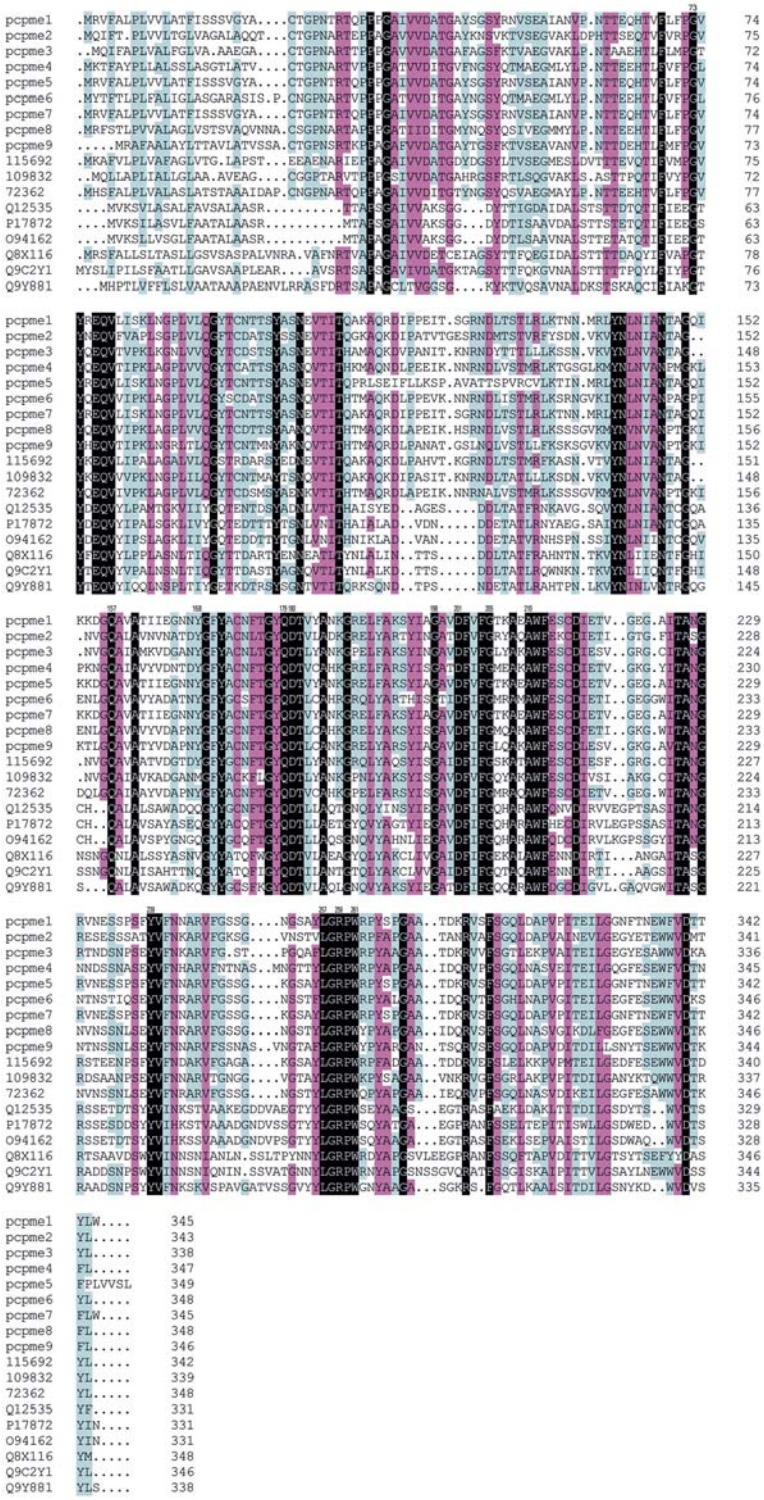

Figure 1. Amino acid sequence alignment of 17 selected PMEs. Five conserved sequence segments (73_GxYxE, 157_QAVAT, 179_QDTV, 201_DFVFG, and 257_LGRPW, numbered according to their positions in *Pcpme*1) exist in all nine *Pcpme* genes. Sequences analyzed included: *Pcpme*1 to *Pcpme*9 (from *P. capsici,* Genbank no: EF596784, FJ213426-33); 115692 and 109832 (from *P. sojae*: http://genome.jgi-psf.org/Physol_1.home.html); 72362 (from *P. ramorum*: http://genome.jgi-psf.org/Phyral_1.download.html); Q12535 (from *Aspergillus aculeatus*); P17872 (from *A. tubingensis*); O94162 (from *A. oryzae*); Q8X116 and Q9C2Y1 (from *Botryotinia fuckeliana*); Q9Y881 (from *Cochliobolus carbonum*). *Pcpme*1 was placed in the first line as leading number. Dark highlights indicate that the residues are conserved in all PMEs compared, whereas other colors highlights denote sequences only conserved in some of PMEs.

Table 3. Nine *Pcpme* genes isolated from *P. casici*.

Genes	GenBank No	Encoding polypeptide	Molecular mass(KDa)	Signal peptide length	Potential N-linked glycosylation	ORF(bp)
Pcpme1	EF596784	345	37.7	20	8	1035
Pcpme2	FJ213426	343	36.9	19	3	1029
Pcpme3	FJ213427	338	36.2	19	4	1014
Pcpme4	FJ213428	347	38.1	20	6	1041
Pcpme5	FJ213429	349	37.9	20	7	1047
Pcpme6	FJ213430	348	38.2	20	4	1044
Pcpme7	FJ213431	345	37.7	20	7	1037
Pcpme8	FJ213432	348	37.8	20	6	1044
Pcpme9	FJ213433	346	37.7	16	7	1038

significant expression level changes from 1 to 5 dpi, and peaked at 7 dpi. The *Pcpme*6 transcripts were highest among the nine *Pcpme* genes in treated fruits at 7 dpi (Fig. 2F), which was ca. 1–6 fold higher than for the other eight genes. *Pcpme*1 and *Pcpme*5 showed relatively high expression levels at 7dpi, over 2–7 fold more than the remaining *Pcpme* genes at the same time point.

Expression of the nine Pcpme genes during infection of tomato fruits

Expression of the nine *Pcpme* genes was investigated at four time points after inoculation (e.g., 1, 3, 5, 7 dpi); detached tomato fruits were treated with the same procedures as that of pepper fruits. It is valuable to note that *Pcpme*1, *Pcpme*3, *Pcpme*4, *Pcpme*5, *Pcpme*6, *Pcpme*7 and *Pcpme*9 genes displayed similar expression patterns, in which expression levels were very low at the first three time points, followed by a great shift, peaking at 7dpi (Fig. 3A, C–G, and I). In contrast, expression levels of *Pcpme*2 and *Pcpme*8 gradually increased from 1 to 3 dpi, then rapidly decreased to minimal levels at 5 dpi (Fig. 3B and H). Notably, the *Pcpme*6 transcripts were similar to those in the pepper fruits, and revealed the highest expression levels among the nine genes in treated tomato fruits (Fig. 3F). *Pcpme*4 expression levels were ranked as second among nine genes (Fig. 3D). The expression patterns of *Pcpme*1 were almost consistent with those of the *Pcpme*9, which were about 1.5–2.5 fold lower than those of *Pcpme*6 at 7 dpi (Fig. 3). And *Pcpme*2, *Pcpme*3, *Pcpme*7 and *Pcpme*8 revealed parallel expression levels at 7 dpi and appeared 2.5–4 fold less than those of both *Pcpme*4 and *Pcpme*6 at 7 dpi. Meanwhile, the value of *Pcpme*5 at 7 dpi was ca. 6 fold less than that of *Pcpme*6 at 7 dpi (Fig. 3).

Expression of the nine Pcpme genes during infection of cucumber fruits

In cucumber fruits, the expression profiles of the nine *Pcpme* genes were significantly different from those observed in both solanaceous plant fruits. Unexpectedly, the expression of each gene was very low at the first two time points compared with the remaining time points. All genes displayed an expression peak at

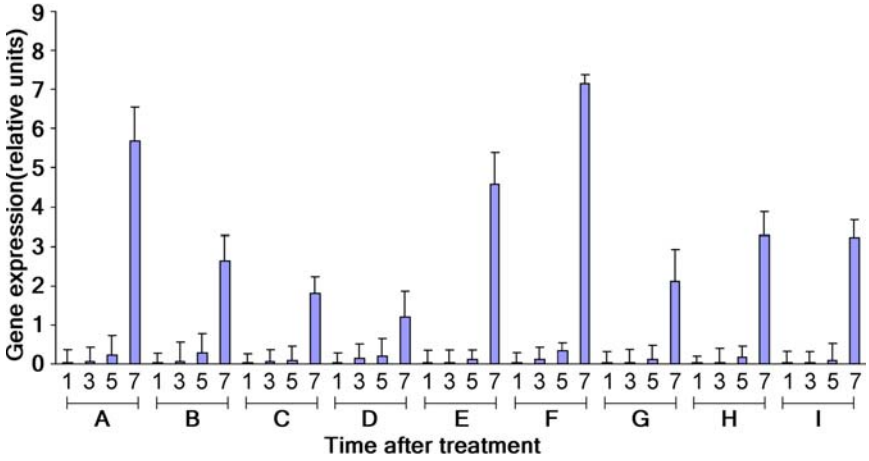

Figure 2. Real-time PCR analysis of nine *Pcpme* gene expression in pepper fruits inoculated with zoospore suspension of *P. capsici* at 1, 3, 5 and 7 dpi. A: *Pcpme*1, B: *Pcpme*2, C: *Pcpme*3, D: *Pcpme*4, E: *Pcpme*5, F: *Pcpme*6, G: *Pcpme*7, H: *Pcpme*8, I: *Pcpme*9. 18S rRNA was chosen as an endogenous control. Data represent the average of three independent experiments with standard errors.

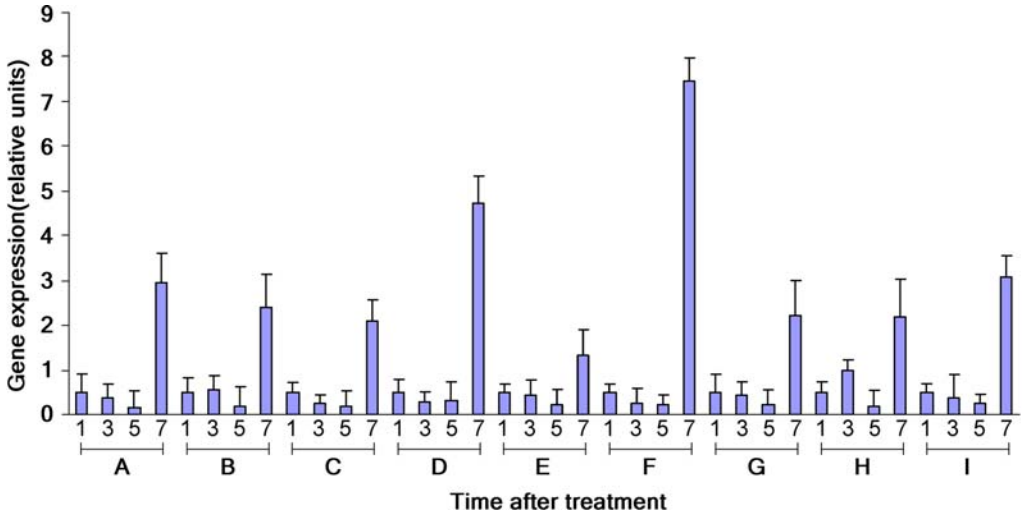

Figure 3. Real-time PCR analysis of nine *Pcpme* gene expression patterns in tomato fruits inoculated with zoospore suspension of *P. capsici* at 1, 3, 5 and 7 dpi. A: *Pcpme*1, B: *Pcpme*2, C: *Pcpme*3, D: *Pcpme*4, E: *Pcpme*5, F: *Pcpme*6, G: *Pcpme*7, H: *Pcpme*8, I: *Pcpme*9. 18S rRNA was chosen as an endogenous control. Data represent the average of three independent experiments with standard error.

5 dpi, and decreased from 5 to 7 dpi (Fig. 4). It was intriguing that *Pcpme*2, *Pcpme*3, *Pcpme*4, and *Pcpme*6 were expressed at low levels in all stages of infection, while *Pcpme*1, *Pcpme*5, *Pcpme*7, *Pcpme*8, and *Pcpme*9 expression levels rapidly reached a peak at 5dpi, and gradually declined at 7 dpi (Fig. 4). The definite peaks of the nine genes were only observed at 5 dpi suggesting that this stage may be critical for the ability of *P. capsici* to successfully secrete the PMEs that cause observable necrotic lesions.

Discussion

We cloned nine *Pcpme* genes by screening a genomic library from highly virulent *P. capsici* strain SD33, and assayed expression patterns of these genes in different hosts including pepper, tomato, and cucumber. Our results showed that individual members of the *Pcpme* gene family (encoding pectin methylesterase) showed differential expression patterns depending on the stage of infection and the hosts. Because *P. capsici* can infect a variety of hosts, we suggested that individual member of the *Pcpme* gene family might play specific roles in infecting different host species. In order to prove involvement in pathogenesis, it is a prerequisite for a gene to be expressed in some stage of the infection process. In order to designate priorities for internuclear gene silencing in *P. capsici* [35], it is essential to have information on the expression of individual member of the *Pcpme* gene family in individual hosts. *Bcpme*1 isolated

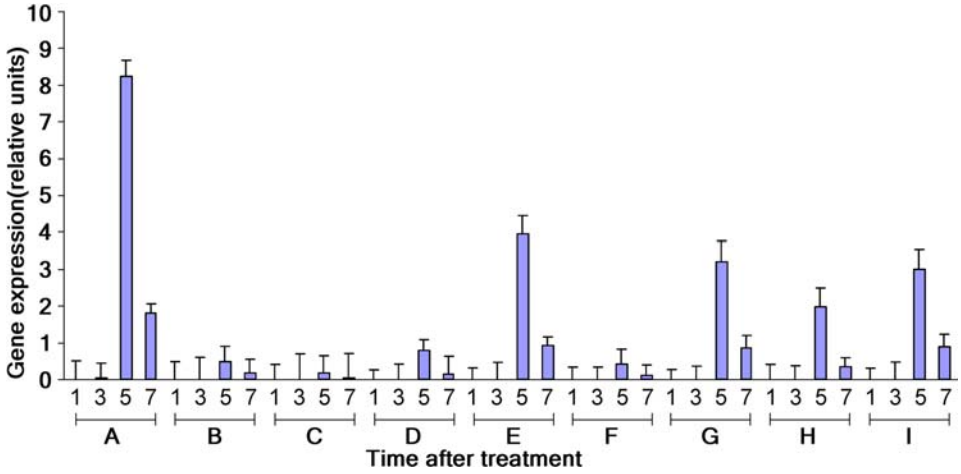

Figure 4. Real-time PCR analysis of nine *Pcpme* gene expression in cucumber fruits inoculated with a zoospore suspension of *P. capsici* at 1, 3, 5 and 7 dpi. A: *Pcpme*1, B: *Pcpme*2, C: *Pcpme*3, D: *Pcpme*4, E: *Pcpme*5, F: *Pcpme*6, G: *Pcpme*7, H: *Pcpme*8, I: *Pcpme*9. 18S rRNA was chosen as an endogenous control. Data represent the average of three independent experiments with standard error.

from *Botrytis cinerea* was shown to be expressed in different host plants [16], and a polygalacturonase gene family of *B. cinerea* was expressed in various plant tissues [36]. However, there is currently no information about members of *Pcpme* gene family expressed during the infection process and shown to be required for full virulence in different hosts.

The nine *Pcpme* genes revealed different expression levels during infection of pepper, tomato and cucumber fruits by *P. capsici*, suggesting that they may play diverse roles in pathogenicity. Prior to the present study, it was not well known that *Pcpme* gene expression diversity occurred at different time points throughout the course of *P. capsici* infection of three different plants. The present study was undertaken to answer this critical question, as expression levels shift of *Pcpme* genes might relate to the symptom expansion in the course of infection.

In analyzing gene expression levels we also noted a surprising dissimilarity of expression patterns among the three different hosts tested (Figs. 2–4). This may reflect that individual members within the *Pcpme* gene family show diversity in gene expression within the wide host range of *P. capsici.* Like the polygalacturonase (PG) gene [36], *Pcpme* genes are variously expressed during the entire infection process of *P. capsici*. Our observations with *P. capsici* indicate similarity with *Erwinia chrysanthemi* [37] in which all *Pme* genes are variously expressed in the systemic phase of the disease. On the other hand, *Pcpme*1, *Pcpme*5 and *Pcpme*6 are more highly expressed in pepper fruits, indicating that pepper may be more suitable for expression of these three genes during interaction with *P. capsici*. By contrast, both *Pcpme*1 and *Pcpme*5 are highly expressed in cucumber fruits, and both *Pcpme*4 and *Pcpme*6 are highly induced in tomato fruits, suggesting that various *Pcpme* genes may respond specifically to different host plant species. Our data support the idea that different members in a gene family may be involved in host specificity [29].

Determining the expression patterns of gene family members may help in the characterization of different genes so that attention may be focused on particular developmental stages or organs where closely related gene family members are not simultaneously expressed [38]. Although members of gene families or 'superfamilies' are grouped together based on a shared motif or domain and consequently, they may have disparate functions. Within gene families some members are often highly expressed, such as four of these nine *Pcpme* genes (*Pcpme*6, *Pcpme*1, *Pcpme*5, and *Pcpme*4), perhaps providing activity at a constitutive level. On the other hand, the other five members are expressed at low levels, possibly only in specific tissues or under more specific conditions.

qPCR experiments showed that all nine *Pcpme* genes were expressed in different plant fruits but differently. Nine *Pcpme* genes showed significantly different expression patterns as did PG genes of *B. cinerea* in various plant tissues [36]. It appeared that some of the genes (*Pcpme*6, *Pcpme*1 and *Pcpme*5 in pepper fruits; *Pcpme*6 and *Pcpme*4 in tomato fruits; *Pcpme*1 and *Pcpme*5 in cucumber fruits) were expressed at very high levels during late phases of infection, compared with the relatively low levels observed for the remaining *Pcpme* genes. The results indicated that these four genes (*Pcpme*6, *Pcpme*1, *Pcpme*5, and *Pcpme*4) might play major roles in modification of pectin in plant cell walls accompanied by PGs and additional pectinases. Additionally, expression levels shifted during different experimental phase, which may indicate a diversity of molecular processes taking place. All nine genes showed low expression levels during early phases of infection, which indicated that corresponding PMEs secretion was low at these time point. Possibly, plant defenses were trigged in initial phases and played a role in minimizing the relative *P. capsici* development and consequently inhibiting PMEs secretion.

In regarding the data on expression of nine *Pcpme* genes in three different plant tissues, we observed that strong expression of *Pcpme*6 at 7dpi was entirely restricted to pepper and tomato fruits (Figs. 2 and 3), which indicates that *Pcpme*6 may play an important role in late stages of infection. It is also possible to infer that *Pcpme*6 may thus play an important role in pathogenicity during *P. capsici* infection of other solanaceous plant host as well. In addition, we can not reject the idea that *Pcpme*1 may be significant in other cucurbitaceae plants exhibiting necrotic lesions during late phases of infection, as *Pcpme*1 showed significant expression levels in cucumber fruits. This illustrates that *Pcpme*6 and *Pcpme*1 can be considered new targets to be further studied in exploration of the pathogenicity mechanisms of PMEs during *P. capsici* interaction with solanaceous or cucurbitaceous plants.

The maximum values of *Pcpme* gene expression levels were always present at 7 dpi in fruits of both solanaceous plants, while it always emerged at 5 dpi in cucumber fruits. However, this diversity has not yet been clearly explained. Previous studies revealed that PMEs was a highly specific enzyme for the D-galacturonan structure and its activity was affected by several factors such as pH, ionic strength and temperature [39]. For example, *Aspergillus niger* PMEs had an optimal pH of 5 for enzymatic activity [40], whereas the pH optimum

for *P. capsici* PMEs was 6.5 [10]. In addition, pectin and pectic acid in plant hosts induced microorganisms to produce PME. Thus, host factors can promote expression of *Pcpme* genes during *P. capsici* infection of plant tissues. The previous study confirmed that both pepper and tomato fruits have higher pectin content than that of cucumber fruits [41] and the fruit tissue pH values are closer to the optimum pH for *P. capsici* PMEs compared with cucumber fruits (data not shown). These data could explain why the nine *Pcpme* genes show stronger expression levels in both solanaceous fruits than in cucumber fruits during the infection process. However, it is not yet clear why the distinct peaks of *Pcpme* gene expression appeared at different timepoints between solanaceous and cucurbitaceous plants (Figs. 2–4). We note that the *Pcpme*1, *Pcpme*5, *Pcpme*7, *Pcpme*8 and *Pcpme*9 products have more potential *N*-linked glycosylation sites on the amino acid sequences than the other *Pcpme* genes in this study, which may affect enzymatic stability, secretion, solubility, or activity [42, 43]. It is possible that these factors may be related to the five genes' higher expression level relative to other *Pcpme* genes in cucumber fruits [37].

The observations of expression patterns of these nine *Pcpme* genes in three different host species enable predictions about the possible contributions of individual genes to virulence. Internuclear gene silencing [35] experiments using these nine *Pcpme* genes can be utilized in the future to analyze virulence on different plants conditioned by individual genes. Due to results of the present study, improved choices can now be made in setting priorities in such *Pcpme* genes silencing studies that could provide more insight into the function(s) of a single gene or a number of genes, and into the concerted action of PMEs in pathogenesis of *P. capsici*.

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